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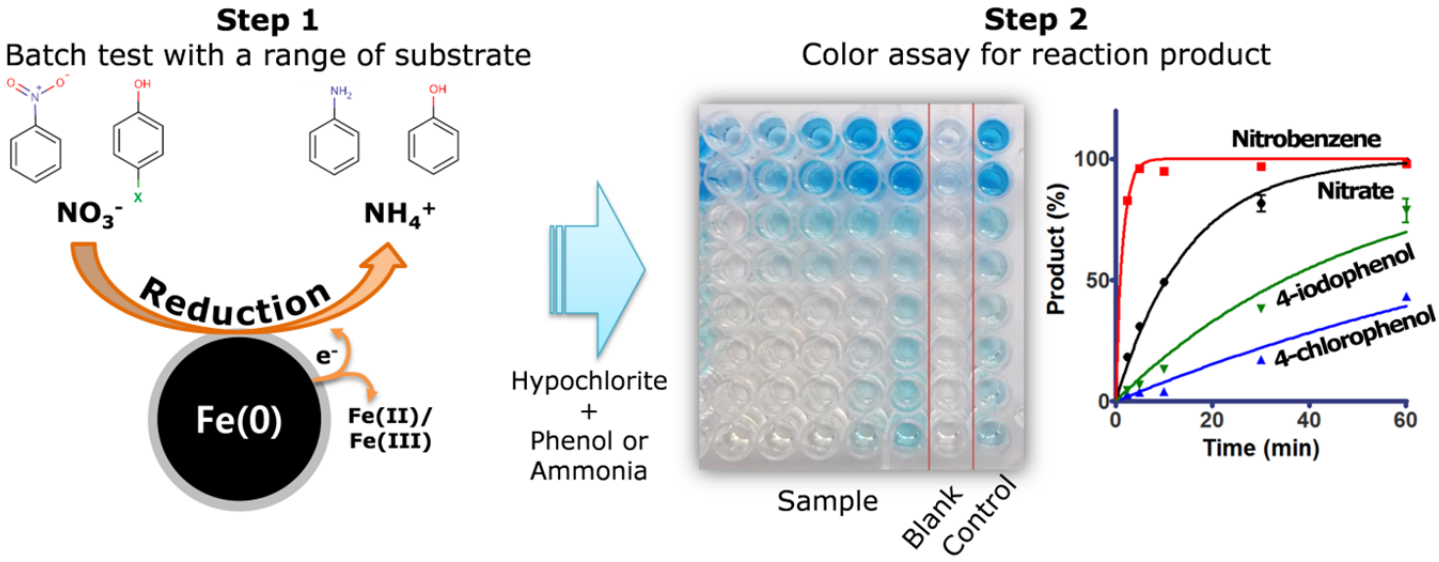
Graduated characterization method using a multi-well microplate for reducing reactivity of nanoscale zero valent iron materials

Yuhoon Hwang[1*], Apostolos Salatas[1,2], Paul D. Mines[1], Mogens H. Jakobsen[3], Henrik R. Andersen[1]

[1] Department of Environmental Engineering, Technical University of Denmark, Miljøvej, B113, DK-2800 Kgs. Lyngby, Denmark
[2] Department of Environment, University of the Aegean, Xenia Building, University Hill, 81100 Mytilene, Greece
[3] Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, B 345E, DK-2800 Kgs. Lyngby, Denmark

Highlights

- Color assays using five substrates characterized a graduated reactivity order of nZVI.
- The assays quantified reduction products to avoid interfering reactions.
- Three reaction products were quantified using both the same wavelength and reagents.
- Assay was adapted to a 96-well microplate, thus minimizing sample and reagent use.



Abstract - Even though nanoscale zero valent iron (nZVI) has been intensively studied for the treatment of a plethora of pollutants through reductive reaction, quantification of nZVI reactivity has not yet been standardized. Here, we adapted colorimetric assays for determining reductive activity of nZVI and its composites with other metals. The assay quantifies reduction products to avoid interfering reactions, such as sorption and volatilization. Three different reaction products, ammonium, phenol, and aniline, generated as the result of reduction of nitrate, p-halophenols, and nitrobenzene, respectively, could be quantified using the same reagent for all reactions. The colorimetric assays were further adapted to the 96-well microplate format, thus minimizing sample and reagent use, as well as lowering color development time to 2 h. The substrates showed graduated reactivity and thus reduction potency and kinetics of different materials and reaction mechanism was distinguished. The applicability was successfully proven by determining the reactivity of a commercial nZVI sample, and investigating the effect of nickel content on dehalogenation. Therefore, the suggested reactivity test with different compounds, combined with the use of a multi-well microplate based color assay, promises to be a useful and simple tool in various nZVI related research topics.

Keywords - Nanoscale zero valent iron; Indophenol reaction; Dehalogenation reactivity; Colorimetric assay; Multi-well microplate

*Corresponding author, Yuhoon Hwang, E-mail:yuoh@env.dtu.dk, Tel: +45-4525-2173, Fax: +45-4593-2850

1. INTRODUCTION

Nanoscale zero valent iron (nZVI) has been intensively studied for environmental remediation, due to its strong reductive power and reactivity, which is derived from its small size and high specific surface area [1]. Its standard redox potential (E^0=-0.44V) makes nZVI an effective material when reacting with reducible contaminants. The removal mechanism by nZVI involves the direct transfer of electrons to the contaminants, transforming them into non-toxic, less toxic, or more biodegradable species [2,3]. A wide variety of pollutants, including halogenated organics, nitro aromatic compounds, heavy metals, and oxy-anions, have been successfully remediated, by utilizing the reduction reaction mechanism [4].

Although a number of different nZVI materials have been prepared and compared, across many intensive studies, different properties of prepared nZVIs make it hard to compare and standardize. Depending on the aim of research and laboratory environment, the synthesis conditions of nZVI can be significantly varied; and, with it brings about considerably different nZVI properties. The effect of synthesis conditions, such as reduction time and precursor concentration, on nZVI characteristics and reactivity has been previously investigated [5]. It has also been determined that nZVI properties can be greatly influenced by solvent conditions during synthesis [6,7]. Moreover, surface modification of nZVI has been intentionally performed, in order to enhance nZVI properties desirable for environmental remediation. In order to prevent aggregation for *in situ* subsurface remediation, particle stabilization or nZVI immobilization on various support materials has been achieved in many studies [8,9]. Recently, remarkable enhancement of stability and reactivity was reported by applying an organo-functionalized clay, i.e. aminoclay [10,11]. Additionally, bimetallic particles with iron as the primary metal and the deposition of a thin layer of noble or transition metals, such as palladium (Pd), copper (Cu), nickel (Ni) or platinum (Pt) on the iron surface has been frequently applied to enhance the reactivity of nZVI, especially for reductive dehalogenation [12–14].

However, the evaluation of nZVI reactivity towards dehalogenation has not yet been standardized. The use of advanced chromatographic techniques has been the predominant method to determine the treatment target and subsequent degradation products [15,16]. These techniques are powerful tools to analyze the compounds, both qualitatively and quantitatively. At the same time, it generally requires lengthy analysis procedures and complicated set-ups. In this light, a simple color assay for use in laboratories, requiring only a basic spectrophotometer for determining color intensity would be a very useful tool.

In our previous study, the modified indophenol method for detecting phenol, which is the result from dehalogenation of 4-chlorophenol, was suggested to determine nZVI reactivity [17]. The aforementioned method was successfully applied to determine dehalogenation reactivity of lab synthesized monometallic and bimetallic nZVI. Throughout the previous study, analysis was focused on determination of the reduction product, rather than the treatment target. This gave a more accurate observation of the reducing activity of nZVI, due to the fact that other reactions, e.g. sorption, oxidation, or co-precipitation cannot interfere. In this study, an extended range of substrates, i.e. nitrate, nitrobenzene, and other halophenols (4-bromophenol and 4-iodophenol), was applied to give better understanding of nZVI reactivity. It was hypothesized that the reduction kinetics and capacity of these substrates would be different, depending on the involved redox reaction and chemical bond. Therefore, the graduated reducing reactivity towards different reducible compounds can be used to determine reduction kinetics and capacity.

Herein, the colorimetric assay to determine nZVI reactivity was further developed and optimized through a multi-well microplate based analysis. Firstly, the previously developed phenol detection method was further miniaturized and optimized for quantification in a 96-well microplate. The expanded range of target compounds, e.g. nitrate, nitrobenzene, and para-positioned halogenated phenols, which can be analyzed by the same reagent to create color, was applied, and subsequently optimized in the same manner. The range of target compounds provided a graduated characterization of reducing activity, with respect to our previously published method using 4-chlorophenol. Lastly, the applicability of the suggested method for investigation on the effect of different factors during the reaction and production of nZVI was demonstrated. Based on the overall results, a simple and effective assay to determine nZVI reactivity is suggested.

2. METHODS

2.1 Color assay for analyzing reaction products

In this study, the same regime of reagents was applied to ammonium, phenol, and aniline to create a colored product. The use of similar reagents gives simplicity and versatility on analyzing several reaction products at the same time. The following sections describe in-detail procedures of the color assays.

2.1.1 Ammonium analysis as a product of nitrate reduction

The indophenol reaction is the name given to the reaction of monochloramine and a phenol, which results in the formation of a blue indophenol dye, which can then be determined spectrophotometrically [18,19]. This reaction is highly specific for ammonium, and in the presence of a suitable catalyst, extremely high sensitivity is attainable. Ammonium analysis was conducted according to standard methods, with a slight modification (4500-NH_3-D) [20] Basically, this consisted of ammonium measurement, using ammonium as a limiting reagent, with excess amounts of phenol and

hypochlorite being applied to ensure all ammonium forms indophenol, when catalyzed by sodium nitroprusside. Reagent A was prepared by diluting 1 mL of 10% hypochlorite solution and 66mL of 0.5M NaOH solution to 100 mL with deionized water. Reagent B was prepared as 26 g/L of phenol and 0.3 g/L of sodium nitroprusside. Samples of 2.3 mL are transferred to a cuvette and 0.1 mL of each reagent is added to the sample. The mixture is then allowed to react in the dark for 2 h and subsequently quantified with a UV-Vis spectrophotometer (Cary 50, Agilent Technologies Inc., USA) at 630 nm.

2.1.2 Phenol analysis as a product of halogenated phenol reduction

In our previous study, phenol detection by the indophenol reaction was successfully developed by simple substitution of phenol as the limiting substrate in the entire reaction [17]. In this color assay, phenol acted as the limiting reagent, and excess amounts of ammonium and hypochlorite were applied, in order to have the color development dependent on the concentration of phenol in samples. For the color assay, Reagent A was modified to have a lower concentration of hypochlorite, which was optimized as described in section 2.2.2. Reagent A was prepared by diluting 0.2 mL of 10% hypochlorite solution and 66mL of 0.5M NaOH solution to 100 mL with deionized water. Reagent C was prepared as 100 mg-NH_4^+-N/L of ammonium chloride and 0.3 g/L of sodium nitroprusside in deionized water. A drop of hydrochloric acid was added into Reagent C to minimize ammonia stripping into the headspace. Equal volumes of reagents A and C were pre-mixed to make the reagent AC. Then, 2.3 mL of sample was transferred into a cuvette, and 0.2 mL of reagent AC was added to the sample. The mixture was allowed to react in the dark for 2 h and subsequently quantified with a spectrometer at 680 nm.

2.1.3 Aniline analysis as a product of nitrobenzene reduction

Reagents A and C were used for aniline analysis, as described in section 2.1.2. In short, 0.1 mL of reagent A was added to 2.3 mL of the sample, and subsequently 0.1 mL of reagent C was added after 15 min of reaction. The mixture allowed to react in the dark for 2 h and then quantified with a spectrometer at 680 nm.

2.2 Miniaturization and optimization for quantification in 96-well microplate.

2.2.1 Use of multiwell plate and plate reader for simple analysis

The multi-well microplate was applied in order to simplify the analysis procedure. Using a multi-well microplate offers the advantage of reading multiple samples with an automated plate reader and having a small sample volume for quantification. In this study, the samples were prepared in a 96-well plate (Nunc polypropylene flat bottom microwell plate, Thermo Fisher Scientific, Inc. USA) and were measured over the range of 400-800 nm, using a Synergy MX microplate reader (BioTek Instruments, Inc., USA). The 96-well plates can hold a maximum volume of approximately 400 µL. In our assays, the volume of the sample was 230 µL and 10 µL for each of the reagents, which is an order of magnitude less volume than when using a normal cuvette. The remaining space in the well was assessed as sufficient for mixing of the mixture.

2.2.2 Optimization of reaction conditions

In order to evaluate the optimal conditions for the reagents, we tested the color assay at different concentration levels of active chlorine for reagent A, 0.01-0.4% active chlorine, through adding 0.1-4 mL of 10% hypochlorite solution in 100 mL reagent A and ammonium for reagent C (50-5000 mg-NH_4^+-N/L). The reaction time was also further optimized by obtaining the calibration curve at different time intervals, up to 24 h. Since ammonium analysis has already been standardized and optimized as a standard method, further optimization was not considered.

2.2.3 Limit of detection and quantification of analytical methods

The calibration curve was acquired by linear regression analysis. The limit of detection (LOD) was calculated as 3 times the standard deviation of the lowest concentration and the limit of quantification (LOQ) as 10 times of the standard deviation of the lowest concentration. Sample absorbance was corrected for reagent blanks and concentrations calculated using a standard curve slope. The tested concentration ranges were (i) for phenol 0-50 µM, (ii) for aniline 0-75 µM and (iii) for ammonium 0-2 mg-NH_4^+-N/L (0-143 µM).

2.3 Preparation of nZVI and bimetallic nZVI

2.3.1 Commercial nZVI particles

Commercially available air-stable nZVI powder (NANOFER STAR) was obtained from Nanoiron s.r.o (Czech Republic). The powder was delivered in an air-tight vial from the manufacturer. Once it was opened, it was stored in an anaerobic chamber (N_2:H_2 = 95:5, Coy laboratory products, USA) to minimize risk of further oxidation.

2.3.2 Preparation of lab synthesized nZVI and bimetallic nZVI

In this study, the mild chemical reduction of metal salts in the solution phase was used to prepare nZVI, as stated in previous research [10]:

$$2Fe^{2+} + BH_4^- + 3H_2O \rightarrow 2Fe^0 + H_2BO_3^- + 4H^+ + 2H_2 \quad \text{(Eq. 1)}$$

The synthesis of nZVI was conducted in a 1000-mL

three-open-neck round bottom flask reactor. The central neck was housed with a tunable mechanical stirrer at 40–2000 rpm (RZR2021, Heidolph, Germany). The stirring speed was adjusted to 250 rpm for nZVI synthesis, and to 100 rpm for aging. Iron precursor solution was prepared with 600 mL of 0.24M $FeSO_4 \cdot 7H_2O$ in deionized water. The reduction of iron ions was instigated by introducing 200 mL of 1.8M $NaBH_4$ solution via peristaltic pump with a constant delivery rate of 10 mL/min. The black precipitate was further aged for 30 min after injection of $NaBH_4$ solution. Collection and washing of nZVI was performed via centrifugation (2500 rpm, 5 min, Heraeus Multifuge X1, Thermo Fisher Scientific Inc., USA), and the collected nZVI particles were then washed once with deionized water and twice with absolute ethanol. The washed nZVI particles were subsequently dried in a vacuum oven at 60°C for 4 h. The dried nZVI was stored in air tight vials inside of the anaerobic chamber. The physical characteristics of was not considered.

prepared nZVI are considered to be identical with uncoated nZVI prepared in previous research [10], which used the same synthesis protocol with this study.

Bimetallic particles were prepared via chemical reduction, similar as with nZVI preparation [21]. The secondary metal solution was introduced to nZVI particles, and then spontaneously bimetallic particles were produced. The bimetallic nZVIs were prepared by mixing the secondary metal solution ($NiSO_4$) with nZVI particles. The nickel content was controlled as 0.01-5% by changing volume of nickel stock solution into reaction vials. After 10 min of redox reaction between the second metal and nZVI, the resulting Ni/Fe bimetallic particles were collected and washed twice with deionized water. Bimetallic particles were prepared and used immediately in the reactivity test; therefore, further drying

2.4 Reduction of compounds by nZVIs

Batch tests for nZVI reactivity were conducted in 50-mL glass serum vials. The desired amount of nZVI was weighed in reaction vials inside of the anaerobic chamber. Then, reaction vials were filled with 39 mL of deionized water and securely capped with a PTFE/silicon septum and aluminum crimp seal. Each stock solution of 1 mL was injected through the septum to initiate the reaction. The nominal concentrations of nitrobenzene, nitrate, para-halogenated phenols and nZVI were 500 μM, 25 mg-NO_3^--N/L, 500 μM, and 2 g-Fe/L, respectively. Vials were mixed on an orbital shaker throughout the entire reaction. Samples of 4 mL were taken periodically and filtered with 0.45 μm syringe filters. The concentration of reaction products in the sample were measured, as described in Section 2.2. Reaction kinetics were further interpreted by pseudo first-order kinetics (Eq. 2).

$$C_t = C_0 \cdot e^{-kt} \quad \text{(Eq. 2)}$$

Where C_t is the concentration at the reaction time, C_0 is the initial concentration, and k is the reaction rate constant (h^{-1}).

3. RESULTS AND DISCUSSION

3.1 Modified indophenol reaction based color assay for analyzing reaction products in multi-well microplate

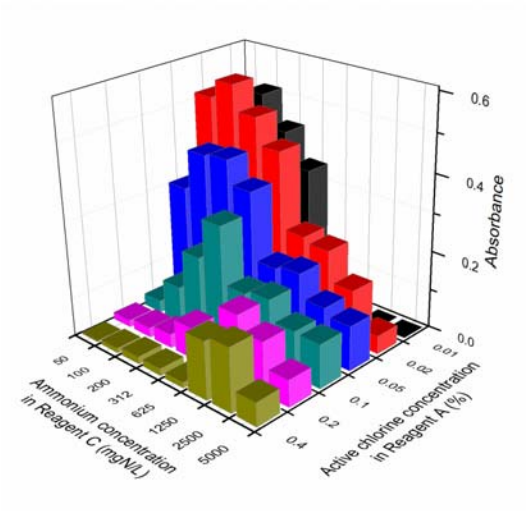
3.1.1 Ammonium analysis

Further optimization on reagent concentration and time for the ammonium analysis was not performed, being that it is already a standardized method. Sample and reagent volumes were decreased an order of magnitude, to fit in the wells of the multi-well microplate. The standard curve, LOD, and LOQ were obtained using the multi-well plate and plate reader, as presented in Figure S1 and Table 1. The values of the LOD and LOQ in 2 h were 2.9 and 9.6 μM, respectively.

3.1.2 Phenol analysis

Effect of the reagent concentration on phenol detection was investigated, as presented in Figure 1 (a). The maximum absorbance was obtained when using 0.02% active chlorine in reagent A and 100 mg-NH_4^+-N/L in Reagent C. It gave a nominal concentration in the mixture of sample and reagent as 9.3 mg/L of active chlorine and 4 mg-NH_4^+-N/L. This clearly demonstrated that the absorbance decreases with increasing amount of hypochlorite and increasing amount of ammonium. The excess hypochlorite will react with phenol causing the decrease of the absorbance. Moreover, more ammonium is required to get a maximum absorbance with increased hypochlorite concentration. A linear relationship could be obtained between the amount of hypochlorite and the amount of ammonium; therefore, it could be concluded that color development is closely related to the stoichiometry of the reaction.

(a)



(b)

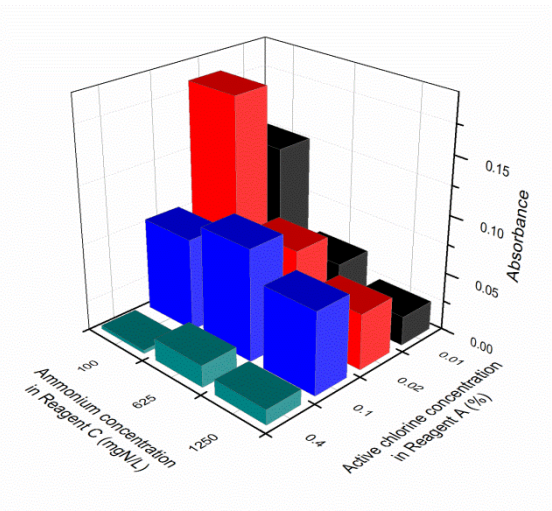


Figure 1 Optimization of reagent concentration on color development for (a) phenol, and (b) aniline. Note that 50 µM of each product was applied and absorbance was measured after 2 h of reaction.

Effect of reaction time was also investigated, as detailed in Figure S1 and Table S1. The slope is steadily increasing during the 2 h of reaction, and it was stable up to 4 h of reaction time. The absorbance then decreased during the remainder of the 24 h reaction time, which indicated that the color product is not stable after 4 h. In summary, the optimal reaction time can be determined as 2 h, since it demonstrated comparably high absorbance and linearity, as well as a shortened reaction time within the stable time range of 2-4 h. The values of the LOD and LOQ in 2 h were 0.3 and 1.0 µM, respectively.

3.1.3 Aniline analysis

The suggested color assay was tested for detection of both aniline and nitrobenzene in a concentration of 50 µM. The adsorption spectra of the color product by aniline and nitrobenzene are detailed in Figure S2. The reaction when applyinganiline produced a color product with a clear peak at a wavelength of 680 nm, regardless of the presence of nitrobenzene. Thus, it was obvious that the selectivity of the color assay adequately distinguished aniline.

Figure 1 (b) outlines how the optimal reagent concentrations for aniline detection are the same as with the phenol assays, as described in section 3.1.2. The sensitivity of color development is lower than with the detection of phenol, therefore, the LOD and LOQ are slightly higher, 0.5 and 1.7 µM for the LOD and LOQ, respectively. Figure S3 and Table S2 present a standard curve optained, with respect to reaction time. The slope is almost stable after a hour of reaction time, which is faster than the reaction with phenol. Since the standard curve slope and its accuracy is nearly stable after a hour of reaction, we took 2 h to be the standard condition, which has the advantage that phenol and aniline can be measured concurrently.

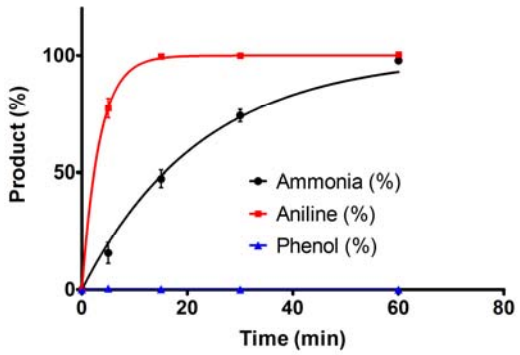
Table 1 Summary of analytical methods using a multi-well microplate for determination of reaction products.

	Ammonium	***Phenol***	***Aniline***
Sample volume (µL)	230	230	230
Reagents	A + B	AC	A+C (15 min interval)
Concentration range for analysis (µM)	0-143 (0-2 mg-NH_4^+-N/L)	0-50	0-75
LOD (µM)	2.9	0.3	0.5
LOQ (µM)	9.6	1.0	1.7
Reaction time (h)	2	2	2

3.2 Graduated characterization of reducing activity by applying a range of reducible compounds

A range of reducible compounds was tested to characterize the graduated reducing activity. In this study, nitrate, nitrobenzene, and para-halogenated phenols (i.e. 4-chlorophenol, 4-bromophenol and 4-iodophenol) were applied. Both lab-synthesized monometallic nZVI and bimetallic nZVI (Ni/Fe 5 % w/w) were applied. The iron concentration was fixed as 2 g-Fe/L.

(a)



(b)

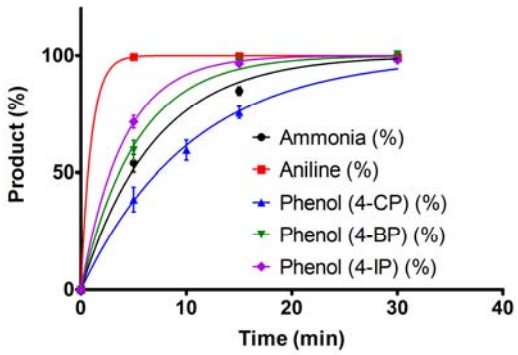


Figure 2 Generation of reaction products by reaction with (a) monometallic nZVI, and (b) bimetallic Ni/Fe **(5%). Note that the iron concentration in all cases was 2 g/L, and curves were fitted by pseudo first-order kinetics. Legends indicate the treatment target and its products.**

Table 2 Kinetic constants and half-life time derived via curve fitting by pseudo first-order kinetics (lab synthesized nZVIs).

nZVI	*Compounds*	*Kinetic constant (h^{-1})*	*$t_{1/2}$ (min)*	*R^2*
Monometallic nZVI	Nitrate	2.65	15.7	1.00
	Nitrobenzene	17.9	2.32	1.00
Bimetallic nZVI (Ni/Fe 5%)	Nitrate	10.3	4.05	0.98
	Nitrobenzene	93.4	0.445	0.99
	4-chlorophenol	5.72	7.27	0.99
	4-bromophenol	10.1	4.11	0.99
	4-iodophenol	14.9	2.79	1.00

The graduated reducing activity of nZVI towards different compounds is presented in Figure 2 and Table 2. Reduction kinetics were well described by a pseudo first-order reaction. The reaction rate constant and half-life time was calculated, as is listed in Table 2. Figure 2 (a) presents the reactivity of monometallic nZVI towards different reducible compounds. As a result of nitrate and nitrobenzene reduction by nZVI, ammonium and aniline could be produced, as described in the following equations (Eq. 3-4).

$$4Fe^0 + NO_3^- + 10H^+ \rightarrow 4Fe^{2+} + NH_4^+ + 3H_2O \quad \textbf{(Eq. 3)}$$

$$C_6H_5NO_2 + 3Fe^0 + 6H^+ \rightarrow C_6H_5NH_2 + 3Fe^{2+} + 2H_2O \quad \textbf{(Eq. 4)}$$

Aniline production was the fastest reaction, and the corresponding rate constant and half-life time were calculated to be 17.9 h^{-1} and 2.32 min, respectively; which is 6.75 times faster than that of nitrate. On the other hand, phenol production was not observed from any of the halogenated phenols. This result agrees with previous findings on dechlorination of chlorinated aromatic compounds by bare ZVI [22].

The overall reactions were greatly accelerated by the second metal, as presented in Figure 2 (b). The 5% Ni/Febimetallic particles exhibited 3.9-5.2 times higher reaction rate constants than monometallic nZVI particles. This indicates that the addition of a second metal on the nZVI surface can promote iron corrosion, due to galvanic effects between nickel and iron. A higher standard redox potential of the second metal would develop a galvanic cell where iron corrosion is accelerated [21]. More electrons can be produced and supplied to reducible compounds, and then a faster reaction can be achieved. In addition, catalytic reactivity of the second metal, nickel was also confirmed by para-positioned halogenated phenols. Phenol was produced as a result of the catalytic reduction of para-positioned halogenated phenols, as described in the following equations (Eq. 5-7) [12]. The clear difference caused by the presence of nickel is the reduction mechanism, which is a formation of reactive hydrogen on the second metal particle surface, followed by dehalogenation and hydrogenation by the reactive hydrogen [12].

$$Fe \rightarrow Fe^{2+} + 2e^- \quad \textbf{(Eq. 5)}$$

$$2H^+ + 2e^- \rightarrow 2H^* \quad \textbf{(Eq. 6)}$$

$$RX + 2H^* \rightarrow RH + HX \quad \textbf{(Eq. 7)}$$

A significant difference in reaction kinetics was also obtained among para-halogenated phenols, depending on the substituted halogens. The reaction was faster in the following order: 4-iodophenol > 4-bromophenol > 4-chlorophenol, the order of which agrees well with the corresponding average bond enthalpies. Average bond enthalpies of C-Cl, C-Br, C-I bond are 339, 276, 240 KJ/mol, respectively. This close relationship explains the reason for different reaction kinetics due to halogen atoms.

In summary, nitrate and nitrobenzene can be used to

determine the general reduction reactivity of nZVI, while catalytic reactivity of the second metal can be determined by para-halogenated phenols. The graduated reducing reactivity towards different reducible compounds is capable of being an indicator for determining and estimating difficulties of the reaction. This graduated reducing reactivity also agrees well with previous related literature on the potential of ZVI for different organic contaminants [3]. Depending on the desired characteristics to quantify, either removal capacity in 5 to 10 min or overall kinetics can be measured.

3.3 Applicability of the method for investigation of reducing power of nZVI samples and reaction conditions

The suggested methods, the reactivity test with different compounds, followed by the colorimetric assay for reduction products using a multi-well microplate, can be widely applied in the field of nZVI study. A number of nZVI samples prepared in various conditions can be tested, and it simply presents the effectiveness of prepared nZVI. Moreover, the effect of reaction conditions on nZVI reactivity can easily be simulated and determined using the suggested methods. Herein, several examples of applying the suggested method are introduced and discussed.

3.3.1 Determining a reactivity of unknown nZVI samples

Several commercial nZVI particles are already available on the market and these are readily used in many remediation studies. The manufacturer may provide a guideline for their usage (e.g. recommended dosage or expected reactivity); however, it may change with respect to different batches or different storage conditions. Therefore, it is sometimes hard to estimate suitable testing conditions. Prior to applying nZVI into a remediation site, verifying the reactivity is essential to estimate appropriate remediation action.

In this study, a commercial nZVI was applied, as received, together with bimetallic Ni/Fe particles prepared using commercial nZVI, in order to accelerate the reduction rate and also to promote catalytic reactivity. Being that the commercial nZVI was delivered several months prior to analysis, current properties should be determined.

As illustrated in Figure 3, commercial nZVI has a negligible reactivity towards all compounds tested. In order to accelerate reaction kinetics and promote catalytic reactivity, commercial Ni/Fe particles were applied towards all compounds in different concentrations, 2 and 10 g-Fe/L. Commercial Ni/Fe particles provide a limited reactivity towards nitrobenzene and nitrate; product concentration was further increased by a higher commercial Ni/Fe particles dosage. However, none of the cases exhibited production of phenol, which indicates that the role of nickel is the acceleration of nZVI oxidation by creating galvanic cells, rather than production of reactive hydrogen, in this case.

Overall, commercial nZVI itself has reactivity towards nitrobenzene and nitrate, but the reactivity is too low to compare with lab-synthesized nZVI. It might be due to oxidation during storage or different surface characteristics caused by different synthesis procedures. It was reported that aging of nZVI was significantly dependent on its preparation method [23] and aging conditions [24]. Therefore, in order to apply the commercial nZVI tested in this study on remediation work, the use of extreme conditions, e.g. much higher iron dosage or acidic conditions, etc. should be considered.

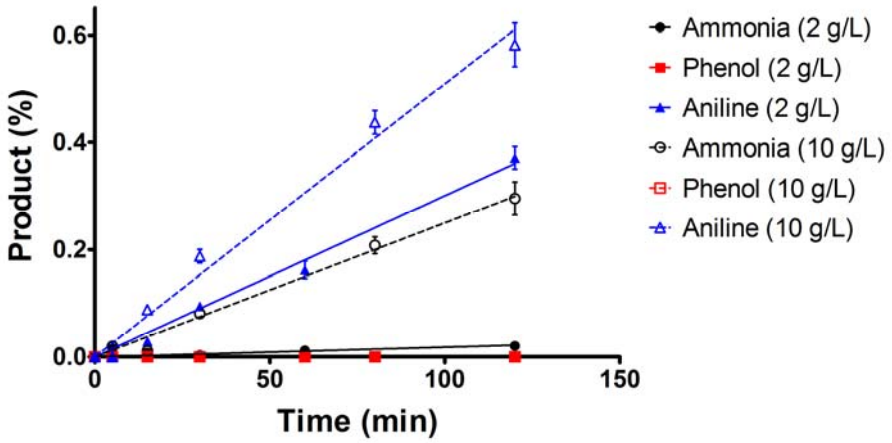


Figure 3 Generation of reaction products by reaction with commercial Ni/Fe**. Note that curves are fitted with pseudo first-order kinetics.**

Table 3 Kinetic constants and half-life time derived by curve fitting via pseudo first-order kinetics (commercial nZVIs).

nZVI	*Compounds*	*Kinetic constant (h^{-1})*	*$t_{1/2}$ (min)*	*R^2*
Commercial Ni/Fe(2 g/L)	Nitrobenzene	0.00181	23000	0.99
Commercial Ni/Fe(10 g/L)	Nitrobenzene	0.00319	13000	0.99
	Nitrate	0.00131	31700	0.99

3.3.2 Effect of bimetal contents on dehalogenation of 4-chlorophenol

Bimetallic particles have been widely applied in order to enhance nZVI reactivity, due to its catalytic behavior and higher standard redox potential promoting iron corrosion. Various transition metals having higher standard redox potentials, e.g. Pd, Pt, Ag, Ni, Cu, etc. have been investigated. However, apart from reactivity enhancement, other aspects should also be considered (e.g. cost of the second metal, acceptable concentration of second metal in receiving water, etc.). Therefore, the effect of bimetallic contents on remediation is often discussed, and optimization is required in many cases.

Herein, the suggested method was applied to investigate the effect of bimetallic contents for dehalogenation of 4-chlorophenol. The nickel content was varied over the range of 0.01-5%. Figure 4 (a) illustrates the reduction of 4-chlorophenol, with respect to time, and rate constants are plotted as Figure 4 (b) in log-scale. Higher Ni content generally brings about faster reaction, which clearly indicates a tremendous effect of hydrogenolysis catalyst on the dechlorination rate. However, the increase of kinetic constants is less significant above 1% of nickel content. This indicates that there is certain ratio between iron and nickel to maximize dehalogenation. When nickel mass loading is too high, the contact area between nickel and iron will be decreased gradually because the ratio of iron becomes too low, which counteracts the reactive hydrogen production, electron transport, catalytic sites, and galvanic couple formation. Similar findings could be found in a study of Cu/Fe bimetallic particles for dechlorination [13,25]. Throughout this study, the effect of nickel contents on dehalogenation was simply and efficiently investigated. Moreover, appropriate remediation conditions could be discussed based on preliminary results showing reaction kinetics and capacity

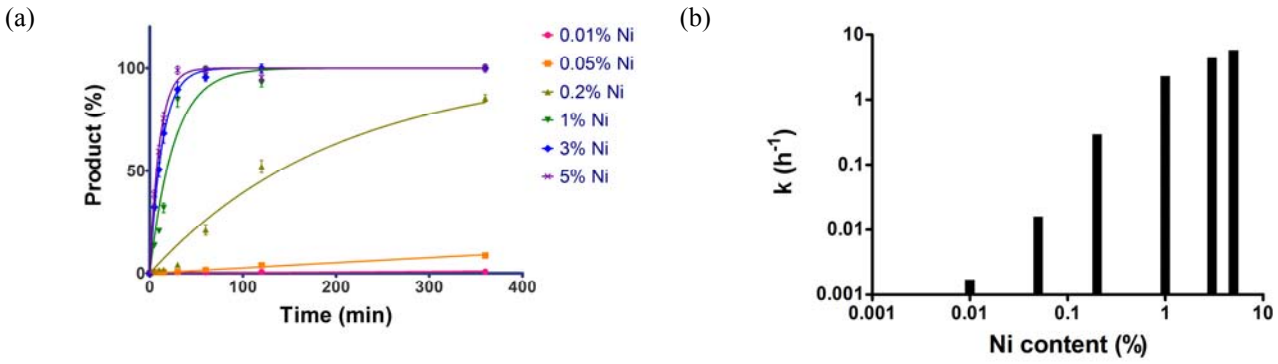


Figure 4 Effect of nickel contents on the 4-chlorophenol reduction by nZVI. (a) Generation of phenol with respect to time, and (b) derived rate constants by curve fitting in log scale. Note that curve was fitted with pseudo first-order kinetics.

3.3.3 Suggested reactivity assay procedure and its implementation

The suggested methods, those of reactivity tests combined with a colorimetric assay using a multi-well microplate, can be widely applied in the field of nZVI studies. It includes, but is not limited to, application of a stabilizer, bimetallic particles, and an electron mediator to enhance nZVI properties for environmental remediation. The overall assay can be performed in two steps: 1) batch tests with a range of substrates, and 2) analysis of reaction products by colorimetric assay, as described in Figure 5. A range of substrates is chosen based on the aim of the study. If the research focuses on investigating catalytic properties or dehalogenation activity of nZVI, a range of halophenols can be applied; whereas nitrobenzene or nitrate can be used to quantify general reduction potential of nZVI. However, it is always a good idea to test at least one of the substrates from each group (e.g. 4-chlorophenol and nitrobenzene), to confirm whether nZVI itself has the reduction capacity and the test conditions are acceptable.

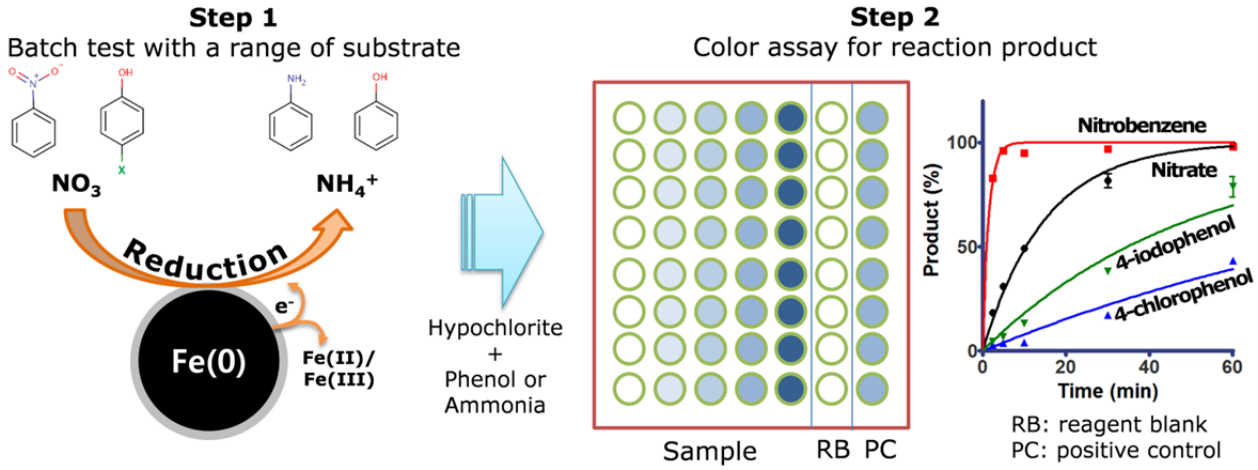


Figure 5 Schematic of the suggested reactivity assay procedure

Figure 5 also explains how the colorimetric assay is designed in a 96-well microplate (12 × 8). Throughout batch tests to measure nZVI reactivity, five samples are taken, up to 1 h of reaction time (e.g., at 2.5, 5, 10, 30, and 60 min). Analysis of samples by the color assay is always done together with a blank, reagent blank, and a positive control spiked with a known concentration of the product, to confirm the validity of the analysis. Thus, it is possible to minimize interference on the colorimetric reaction caused by constituents of the sample, e.g. buffer used or soluble metals. When the soluble iron concentration in a sample is higher than 100 mg/L, sample pretreatment using sodium carbonate solution is considered, as described in previous research [17]. Therefore, the number of wells required for one analysis will be twelve, which fits with a number of wells in a row in the microplate. Several columns can be used according to the number of substrates used for the batch tests and number of replications performed. The color intensity can be quantified at the same wavelength, 680 nm. In the standard analysis for ammonium (see section 2.1.1), the optimal wavelength for ammonia analysis was 630 nm, but it was beneficial to use 680 nm as a universal quantification wavelength, since ammonia showed a much higher color intensity with respect to phenol or aniline in the concentration ranges suggested in Table 1. Therefore, the use of a microplate gives simplicity and versatility for measuring nZVI reactivity, since all analyses, including pretreatment and replication can be performed in a 96-well microplate.

4. Conclusion

The modified indophenol assay for determining the reductive reactivity of nZVI has been suggested. The color reaction for three different reaction products, ammonium, phenol, and aniline are simple and versatile, since the same types of reagents can be applied for all reactions. All color reactions were miniaturized into multi-well microplate, which provides a convenient and efficient assay platform. Reaction conditions (i.e. reaction time and reagent concentrations) were further optimized. A small quantity, 230 µL, is enough to quantify reaction products within 2 h of reaction time.

The three groups of compounds, nitrate, nitrobenzene, and p-halophenols, produced a graduated reactivity order, and it was possible to distinguish the reaction mechanism between normal reduction and that of the catalytic behavior of the second metal. Reaction kinetics were fast enough to complete the reaction within 1 h. Depending on the desired characteristics to quantify, either removal capacity in 5 to 10 min or overall kinetics, can be selectively measured. The applicability was successfully proven by determining reactivity of an unknown nZVI sample and investigating the effect of Ni content with nZVI on dehalogenation. The preliminary results of nZVI reactivity obtained by the suggested assay indicate it is useful to determine suitable reaction conditions for remediation work and to estimate nZVI performance and required time. Therefore, the suggested reactivity test with different compounds, combined with a multi-well microplate based color assay, will be a useful and simple tool in various nZVI related research topics.

Acknowledgement

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Supplementary Information

Graduated characterization method using a multi-well microplate for reducing reactivity of nanoscale zero valent iron materials

Yuhoon Hwang[1,*], Apostolos Salatas[1,2], Paul D. Mines[1], Mogens H. Jakobsen[3], Henrik R. Andersen[1]

[1] Department of Environmental Engineering, Technical University of Denmark, Miljøvej, B113, DK-2800 Kgs. Lyngby, Denmark

[2] Department of Environment, University of the Aegean, Xenia Building, University Hill, 81100 Mytilene, Greece

[3] Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, B 345E, DK-2800 Kgs. Lyngby, Denmark

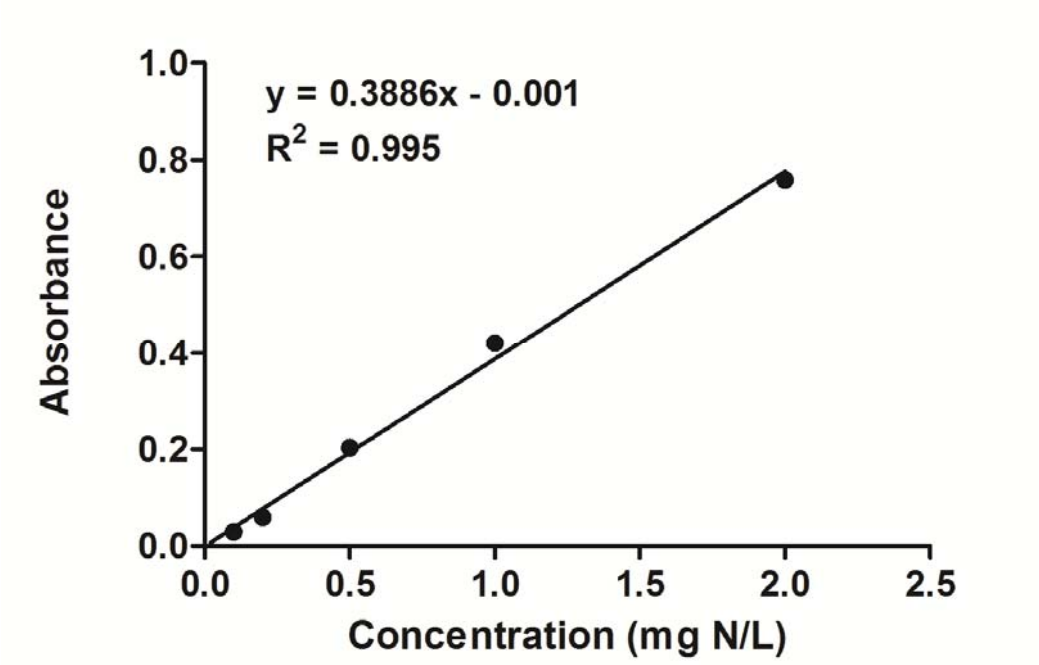

Figure S1 Standard curve for ammonium (0-2 mg-N/L) after indophenol reaction (Reaction time: 2 h).

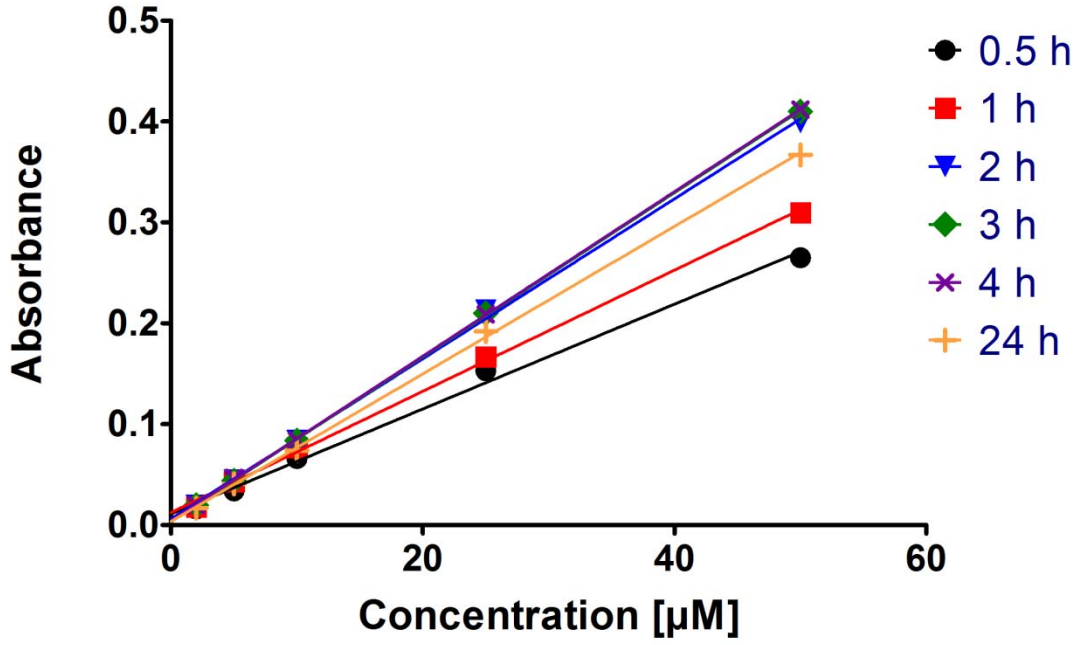

Figure S2 Standard curve for phenol (0-50 μM) after reaction with NH_2Cl (reagent AC) in different reaction time.

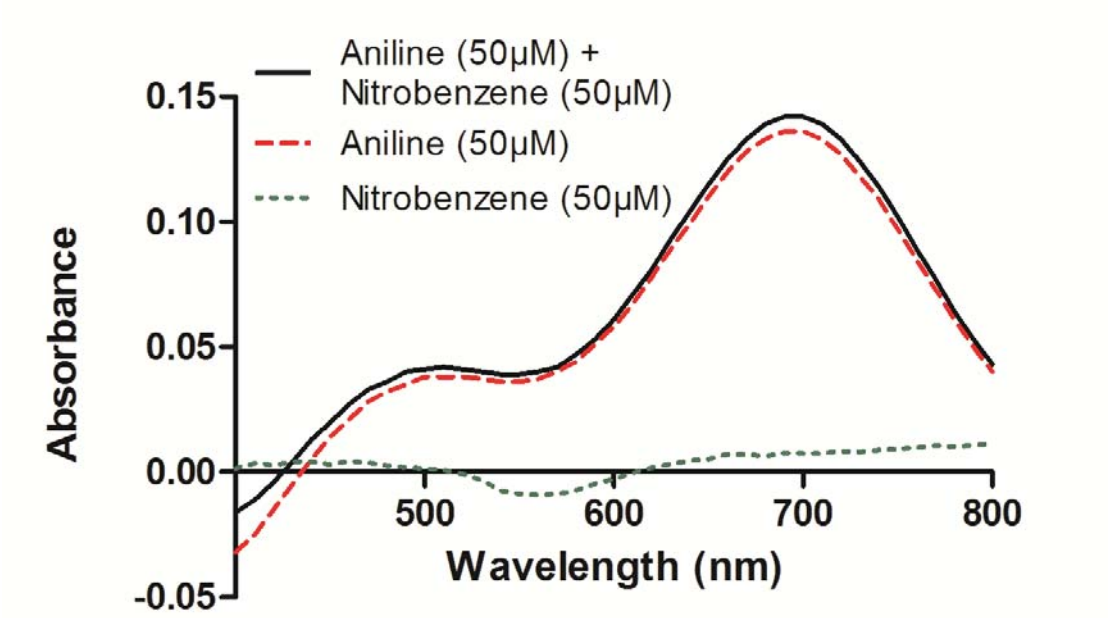

Figure S3 Spectra evolution after color reaction with reagent A and C

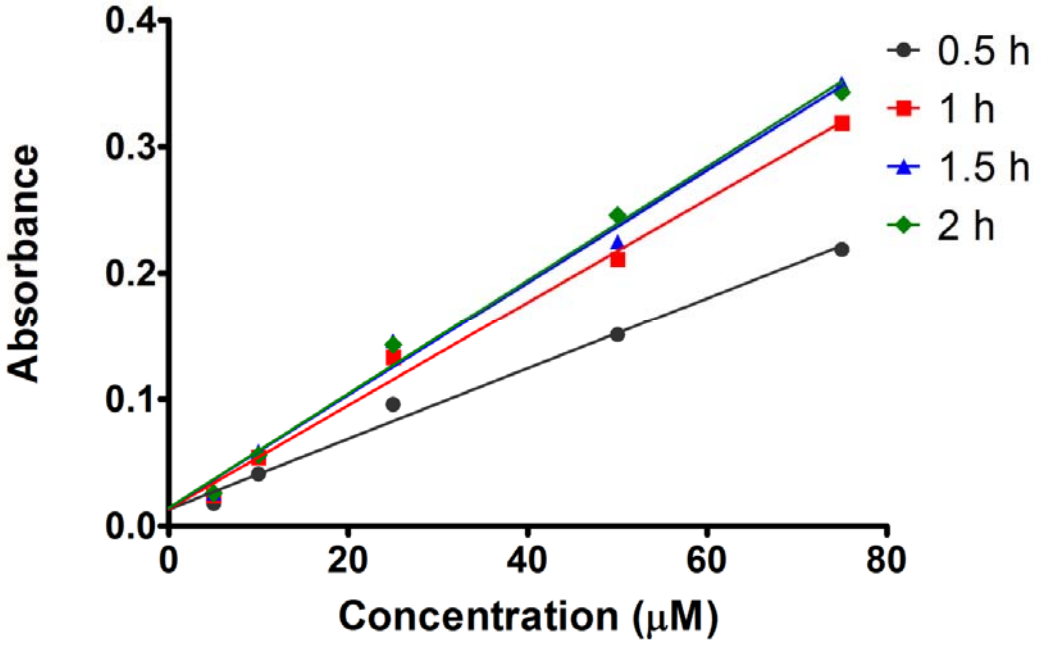

Figure S4 Standard curve for aniline (0-75 μM) after reaction with reagent A and C in different reaction time.

Table S1 Equation, LOD, and LOQ of phenol standard curves in different reaction time

Time (h)	*Standard curve*	R^2	*LOD (μM)*	*LOQ (μM)*
0.5	y = 0.0052x+0.0112	1.00	0.5	1.7
1	y = 0.006x+0.0124	1.00	0.4	1.4
2	y = 0.0079x+0.0063	1.00	0.3	1.1
3	y = 0.0081x+0.0037	1.00	0.3	1.1
4	y = 0.0082x+0.0043	1.00	0.3	1.1
24	y = 0.0073x+0.0037	1.00	0.4	1.2

Table S2 Equation, LOD, and LOQ of aniline standard curves in different reaction time

Time (h)	*Standard curve*	R^2	*LOD (μM)*	*LOQ (μM)*
0.5	y = 0.0028x+0.0131	0.99	1.6	5.4
1	y = 0.0041x+0.0134	0.99	0.9	3.1
1.5	y = 0.0045x+0.0143	0.99	1.3	4.3
2	y = 0.0045x+0.0123	0.99	1.0	3.3